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Original Article

Tanshinone IIA reduces oxidized low-density lipoprotein-induced inflammatory responses by downregulating microRNA-33 in THP-1 macrophages

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Abstract: Atherosclerosis is a leading cause of cardiovascular diseases. Oxidized low-density lipoprotein (ox-LDL) is commonly used to construct atherosclerosis cell models. Macrophages-secreted pro-inflammatory factors play vital roles in the development of atherosclerosis. Tanshinone IIA (Tan) is an effective therapeutic agent for atherosclerotic cardiovascular diseases. However, the molecular mechanisms by which Tan protects against atherogenesis have not been thoroughly elucidated. In the present study, we aimed to search for microRNA targets of Tan in ox-LDL-stimulated macrophages. Interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α) levels were determined by matching ELISA commercial kits. RT-qPCR assay was conducted to measure microRNA-33 (miR-33) expression. We found that ox-LDL induced the secretion of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and the expression of microRNA-33 (miR-33) in THP-1 macrophages. Tan inhibited pro-inflammatory cytokine secretion and miR-33 expression in ox-LDL-stimulated THP-1 macrophages. Also, the depletion of miR-33 suppressed pro-inflammatory cytokine secretion in ox-LDL-stimulated THP-1 macrophages. Moreover, miR-33 upregulation abrogated the inhibitory effect of Tan on pro-inflammatory cytokine secretion in ox-LDL-stimulated THP-1 macrophages. In conclusion, Tan inhibited ox-LDL-induced pro-inflammatory cytokine secretion by downregulating miR-33 in THP-1 macrophages, hinting that Tan might exert its atheroprotective effects by targeting miR-33 and reducing pro-inflammatory responses.

Keywords: Tanshinone IIA, oxidized low-density lipoprotein, microRNA-33, macrophages, inflammation, atherosclerosis

Introduction

Atherosclerosis is the common pathogenesis of vascular diseases including ischemic heart disease (IHD), ischemic stroke, and peripheral arterial disease (PAD), bringing about massive social and economic burden for individuals, families, and society worldwide [1-3]. Mounting evidences show that the dysregulation of immunity and inflammation is closely associated with the pathophysiology of atherosclerosis [4, 5]. Moreover, the accumulation of immune cells such as monocytes/macrophages and lymphocytes within the arterial wall is a major event in atherosclerosis [6, 7]. Macrophages, differentiated from monocytes, have been identified as vital immune cells in all stages of atherosclerosis development, including lesion initiation, lesion expansion, and the formation of advanced plaques [8]. Oxidized low-density lipoprotein (ox-LDL) functions as a major risk factor in the initiation and development of atherosclerosis by inducing a series of atherogenic responses of vascular wall cells (endothelial cells, macrophages, and smooth muscle cells) [9, 10]. Moreover, ox-LDL can be taken up by macrophages, resulting in foam cell formation and inflammatory factor production [10, 11].

Tanshinones are the major effective components of the Chinese medicinal herb Danshen (*Salvia miltiorrhiza*), which has been widely used in Asian countries to prevent and treat cardiovascular diseases (CVD) including athero-

sclerosis, cardiac injury and hypertrophy [12, 13]. Tanshinone IIA (Tan) is one of the most abundant bioactive constituents among the Tanshinones, possessing a lot of pharmacological activities such as anti-oxidation, anti-inflammation, anti-atherosclerosis, and cardio-cerebrovascular protection [13-15]. For instance, Tan could reduce atherosclerotic lesion size and enhanced atherosclerotic plaque stability in Apolipoprotein-E knockout ($ApoE^{-/-}$) mice [16-18]. Tan inhibited atherosclerosis progression by enhancing non-amyloidogenic processing of amyloid precursor protein in platelets [19]. Moreover, Tan is a multi-target drug that can exert its therapeutic effects by regulating various molecules such as scavenger receptors, miRNAs, and inflammatory mediators [20, 21]. For example, Tan attenuated *Porphyromonas gingivalis*-induced atherosclerosis by reducing expression of miR-146b, miR-155, and pro-inflammatory cytokines (*e.g.* interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α)) in Apolipoprotein E knockout mice [22].

In the present study, we aimed to investigate the miRNA targets of Tan in ox-LDL-excited macrophage inflammatory injury.

Materials and methods

THP-1 macrophage culture

Human THP-1 monocytes were purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium (Thermo Fisher Scientific, Rockford, IL, USA) supplemented with 10% FBS (Thermo Fisher Scientific). THP-1 monocytes were stimulated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-aldrich, St. Louis, MO, USA) for 72 h to differentiate into macrophages.

THP-1 macrophage transfection and treatment

THP-1 macrophages were transfected with or without miRNA mimic or inhibitor using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA) following the protocols of manufacturer in the presence or absence of Tan (Sigma-aldrich) stimulation. Then, transfected or/and Tan-treated cells were treated with ox-LDL (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) for corresponding time.

RT-qPCR assay

Total RNA was isolated from macrophages using TRIzol® reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Then, miR-33 expression level was measured using TaqMan MicroRNA assay system (Thermo Fisher Scientific) with U6 snRNA as the internal control.

Enzyme-linked immunosorbent assay (ELISA)

Secretion levels of cytokines such as IL-1β, IL-6 and TNF-α were determined by ELISA assay. Briefly, at the indicated time points after transfection or/and stimulation, cell supernatants were collected and the levels of cytokines in the supernatants were measured by corresponding ELISA commercial kits (Thermo Fisher Scientific).

Statistical analysis

All results were obtained from at least 3 independent experiments and presented as mean values ± standard deviation (SD). Data analysis was performed using one-way ANOVA or Student's *t*-test on GraphPad Prism software (La Jolla, CA, USA). $P < 0.05$ represented the difference was statistically significant.

Results

ox-LDL stimulation induced pro-inflammatory responses in THP-1 macrophages

The ELISA assay showed that ox-LDL treatment at the concentration range of 10-20 μg/ml induced IL-1β, IL-6 and TNF-α secretion in a dose-dependent manner in THP-1 macrophages (**Figure 1A-C**). The treatment of 20 μg/ml ox-LDL resulted in a time-dependent increase of IL-1β, IL-6 and TNF-α levels in THP-1 macrophages (**Figure 1D-F**). In a word, these data revealed that ox-LDL could enhance pro-inflammatory responses in THP-1 macrophages.

Tan alleviated ox-LDL-triggered pro-inflammatory responses in THP-1 macrophages

Next, we further demonstrated that the introduction of 10 μM Tan and 20 μM Tan markedly suppressed the secretion of IL-1β, IL-6 and TNF-α in ox-LDL-stimulated THP-1 macrophages

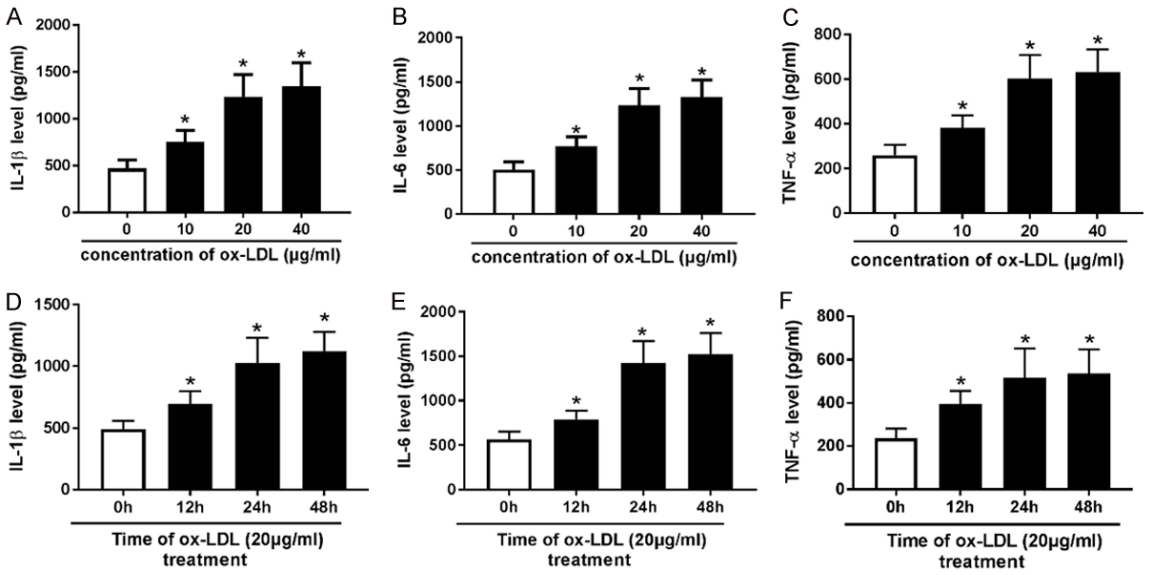

Figure 1. ox-LDL stimulation induced pro-inflammatory responses in THP-1 macrophages. A-C. THP-1 macrophages were exposed to different concentrations (0, 10, 20, 40 μg/ml) of ox-LDL for 24 h. Then, IL-1β, IL-6 and TNF-α levels in cell supernatant were determined by ELISA assay. D-F. THP-1 macrophages were treated with 20 μg/ml of ox-LDL for different time. At the indicated time points (0, 12, 24, 48 h) after treatment, ELISA assay was conducted to measure IL-1β, IL-6 and TNF-α levels. *$P < 0.05$.

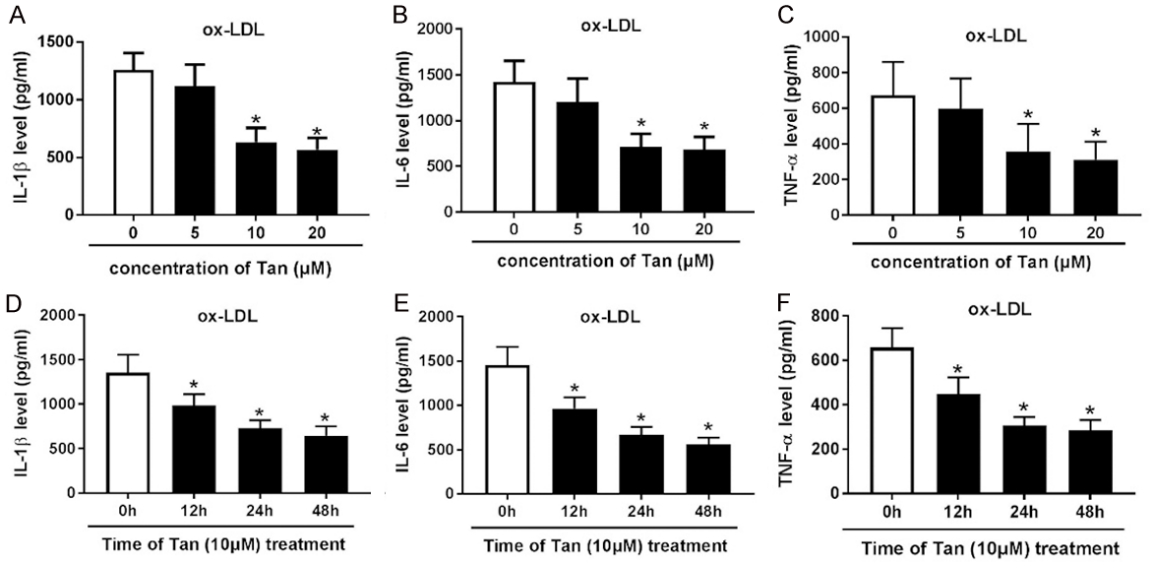

Figure 2. Tan alleviated ox-LDL-triggered pro-inflammatory responses in THP-1 macrophages. A-C. THP-1 macrophages were pre-treated with Tan (0, 5, 10, 20 μM) for 24 h, and then stimulated with 20 μg/ml of ox-LDL for 24 h, followed by the measurement of IL-1β, IL-6 and TNF-α levels through ELISA assay. D-F. THP-1 macrophages were pre-treated with 10 μM of Tan for 0, 12, 24, or 48 h, and then treated with 20 μg/ml of ox-LDL for 24 h. Next, IL-1β, IL-6 and TNF-α levels were detected by ELISA assay. *$P < 0.05$.

(**Figure 2A-C**). Moreover, a reduction of IL-1β, IL-6 and TNF-α levels was noticed in ox-LDL-treated THP-1 macrophages following the introduction of Tan (10 μM) (**Figure 2D-F**). That was to say, Tan could relieve ox-LDL-induced pro-inflammatory responses in THP-1 macrophages.

Tan inhibited ox-LDL-induced miR-33 expression in THP-1 macrophages

Moreover, ox-LDL induced miR-33 expression in a dose- and time-dependent fashion in THP-1 macrophages (**Figure 3A** and **3B**). Additionally, the introduction of Tan resulted in a dose- and

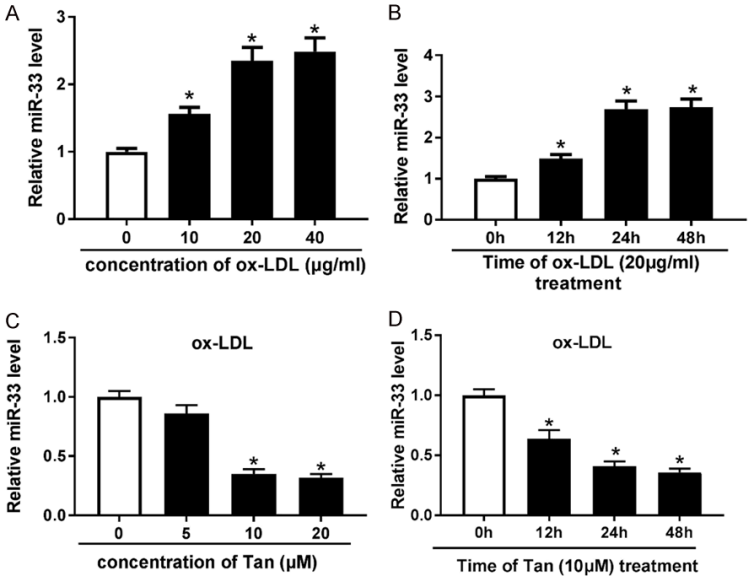

Figure 3. Tan weakened the stimulatory effect of ox-LDL on miR-33 expression in THP-1 macrophages. A. THP-1 macrophages were treated with different concentrations (0, 10, 20, or 40 μg/ml) of ox-LDL for 24 h. Then, miR-33 level was examined by RT-qPCR assay. B. THP-1 macrophages were exposed to 20 μg/ml of ox-LDL for different time. At the indicated time points (0, 12, 24, or 48 h) after ox-LDL treatment, RT-qPCR assay was carried out to measure miR-33 expression. C. THP-1 macrophages were pre-treated with different doses (0, 5, 10, 20 μM) of Tan for 24 h and then stimulated with 20 μg/ml of ox-LDL for another 24 h, followed by the determination of miR-33 level by RT-qPCR assay. D. THP-1 macrophages were pre-treated with 10 μM of Tan for 0, 12, 24, or 48 h and then induced with 20 μg/ml of ox-LDL for 24 h. Then, miR-33 level was detected by RT-qPCR assay. *$P < 0.05$.

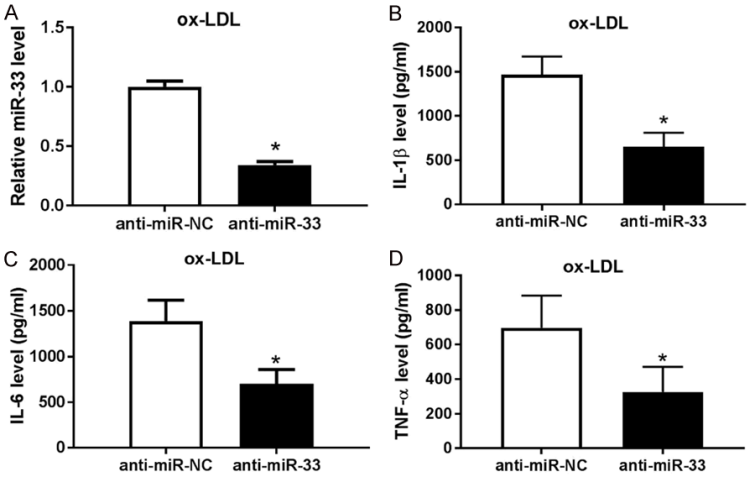

Figure 4. The deficiency of miR-33 reduced ox-LDL-triggered pro-inflammatory responses in THP-1 macrophages. (A-D) THP-1 macrophages were transfected with anti-miR-NC or anti-miR-33 for 24 h and then treated with 20 μg/ml of ox-LDL for additional 24 h. (A) Then, miR-33 level was determined by RT-qPCR assay. (B-D) IL-1β (B), IL-6 (C), and TNF-α (D) levels were measured by ELISA assay. *$P < 0.05$.

time-dependent downregulation of miR-33 level in ox-LDL-treated THP-1 macrophages (**Figure 3C** and **3D**), indicating that Tan abated the stimulatory effect of ox-LDL on miR-33 expression in THP-1 macrophages.

The depletion of miR-33 reduced ox-LDL-triggered pro-inflammatory responses in THP-1 macrophages

Then, transfection efficiency analysis revealed that the transfection of miR-33 inhibitor resulted in the significant downregulation of miR-33 level in ox-LDL-treated THP-1 macrophages (**Figure 4A**). Moreover, the deficiency of miR-33 suppressed the secretion of IL-1β, IL-6 and TNF-α in ox-LDL-treated THP-1 macrophages (**Figure 4B-D**). Combined with above results, our data suggested that the depletion of miR-33 reduced ox-LDL-induced pro-inflammatory responses in THP-1 macrophages.

Tan weakened ox-LDL-induced pro-inflammatory responses by downregulating miR-33 in THP-1 macrophages

Next, RT-qPCR assay further unveiled that miR-33 level was markedly increased in THP-1 macrophages co-treated with Tan and ox-LDL following the introduction of miR-33 mimic (**Figure 5A**). Moreover, miR-33 upregulation abrogated the inhibitory effect of Tan on secretion of IL-1β, IL-6 and TNF-α in ox-LDL-stimulated THP-1 macrophages (**Figure 5B-D**), meaning that Tan protected THP-1 macrophages from ox-LDL-induced inflammatory damage by downregulating miR-33.

Discussion

Atherosclerosis is a chronic arterial disease that involves multiple cellular and acellular pro-

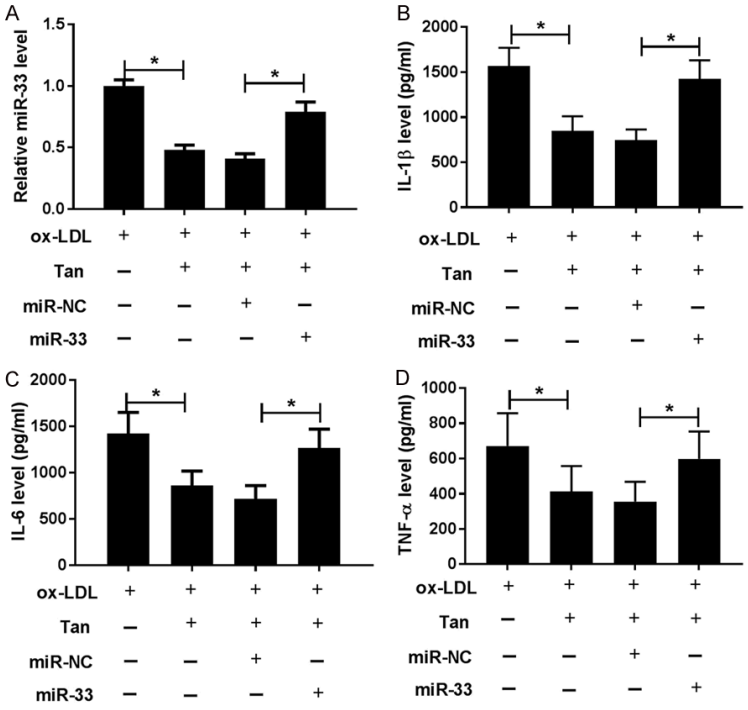

Figure 5. Tan suppressed ox-LDL-induced pro-inflammatory responses by downregulating miR-33 in THP-1 macrophages. (A-D) THP-1 macrophages were transfected with miR-NC or miR-33 mimic for 24 h in the absence or presence of Tan (10 μM), and then treated with 20 μg/ml of ox-LDL for 24 h. (A) Next, miR-33 level was detected by RT-qPCR assay. (B-D) IL-1β (B), IL-6 (C), and TNF-α (D) levels were determined by ELISA assay. **P* < 0.05.

cesses including macrophage polarization, inflammatory factor generation, oxidative stress response, and plaque rupture [23]. Moreover, copious data show that the activation of inflammation and high expression of inflammatory cytokines can aggravate the development of atherosclerosis [24].

Previous studies showed that Tan could prevent the initiation and progression of atherosclerosis by regulating cholesterol metabolism, inflammatory and oxidative responses, along with the development of macrophages and vascular smooth muscle cells (VSMCs) [18, 22, 25]. For instance, Liu *et al.* pointed out that Tan stimulation resulted in the downregulation of macrophage content and cholesterol accumulation, and the inhibition of atherosclerotic plaque development in apolipoprotein E-deficient mice [26]. Moreover, Tan reduced ox-LDL uptake, inhibited ox-LDL-induced foam cell formation and increased cholesterol efflux in human and mouse macrophages [26, 27]. Tan suppressed ox-LDL-induced apoptosis of VSMCs and ox-LDL-induced proliferation and migration of RAW264.7 mouse macrophage cells [18].

IL-1β, IL-6 and TNF-α have been identified as pro-atherogenic cytokines and can be secreted by lymphocytes, natural killer cells, macrophages and vascular smooth muscle cells [28]. In the present study, we aimed to further explore the roles and molecular basis of Tan on pro-atherogenic cytokine secretion in ox-LDL-stimulated THP-1 macrophages.

Our data showed that Tan attenuated ox-LDL-induced secretion of pro-inflammatory factors (IL-1β, IL-6 and TNF-α) in human macrophages, indicating that Tan could mitigate ox-LDL-induced inflammatory injury. Consistently, previous studies showed that Tan inhibited the production of pro-inflammatory cytokines (including IL-1β, IL-6 and TNF-α) in ox-LDL-stimulated RAW264.7 THP-1 macrophages and the serum of ApoE$^{-/-}$ mice [16, 18]. Also, Ma *et al.* showed that Tan inhibited the production of IL-1β and TNF-α in LPS-stimulated THP-1 macrophages [29].

MicroRNAs (miRNAs), a group of small non-coding RNA transcripts with the length of about 22 nucleotides, have emerged as vital modulators of ox-LDL-mediated signals in different vascular cells including macrophages [30]. For instance, the ectopic expression of microRNA-212 facilitated lipid accumulation and inhibited ATP binding cassette subfamily A member 1 (ABCA1)-dependent cholesterol efflux by targeting sirtuin 1 (SIRT1) in ox-LDL-treated THP-1 macrophages [31]. MicroRNA-181a overexpression suppressed ox-LDL-induced foam cell formation, cell apoptosis, and pro-inflammatory factor expression by targeting toll like receptor 4 (TLR4) in THP-1 macrophages [32]. Moreover, Fan *et al.* unveiled that Tan could exert its anti-inflammatory activity by inactivating TLR4/MyD88/NF-κB signaling pathway and regulating some cytokine and miRNA expression in LPS-induced RAW264.7 cells [33]. Hence,

miRNA targets of Tan were further investigated in ox-LDL-treated THP-1 macrophages. A prior report pointed out that Tan could inhibit lipid deposition and microRNA-33a (miR-33a) expression in the livers of hyperlipidemic rats [34], indicating that Tan might can exert its function by targeting miR-33.

MiR-33, located in the introns of sterol-regulatory element-binding protein (SREBP) genes, has been reported to be a player in multiple vital biological processes such as inflammation [35], metabolism [36] , and mitochondrial function [37]. Moreover, the depletion of miR-33 could inhibit the development of atherosclerosis [38-41]. Additionally, the deficiency of miR-33 induced the polarization of macrophages from an M1 to an M2 phenotype and attenuated lipid accumulation along with inflammatory responses in macrophages [41, 42]. Sun *et al.* further showed that endoplasmic reticulum (ER) stress induced lipid metabolism disorder of macrophages by regulating miR-33 in atherosclerosis [43], hinting the close link between miR-33 and macrophage function in atherosclerosis.

Our present study showed that ox-LDL could induce the notable upregulation of miR-33 in macrophages, while this effect was weakened by Tan. Also, miR-33 loss inhibited the secretion of pro-inflammatory cytokines (including IL-1β, IL-6 and TNF-α) in ox-LDL-stimulated macrophages. Moreover, miR-33 overexpression reversed the inhibitory effect of Tan on pro-inflammatory cytokine secretion in ox-LDL-stimulated macrophages.

Taken together, our data disclosed that Tan alleviated ox-LDL-induced inflammatory injury by targeting miR-33 in human macrophages, providing a novel miRNA target of Tan for the treatment of atherosclerosis and deepening our understanding on the etiology of Tan. However, our study only showed that Tan exerts atheroprotective effects by regulating miR-33. It is necessary to further dig out downstream targets of miR-33. Moreover, *in vivo* experiments were also indispensable to further validate our conclusion. Other indicators of atherosclerosis need to be further examined.

Disclosure of conflict of interest

None.

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